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Ballistic delivery of dyes for structural and functional studies of the nervous system

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Abstract

This chapter describes a detail protocol for rapid labeling of cells in a variety of preparations by means of particle-mediated ballistic (gene gun) delivery of fluorescent dyes. This method has been used for rapid labeling of cells with either lipid or water-soluble dyes in a variety of preparations. In particular, carbocyanine lipophilic dyes such as DiI have been used to obtain Golgi-like labeling of neurons and glia in fixed and live cell cultures, brain slices, as well as fixed post-mortem human brain. Water-soluble calcium indicators such as calcium green-1 dextran have been used to image calcium dynamics in living brain slices and retinal explants. This ballistic labeling technique is thus useful for studying the structure and function of neurons and glia in both living and fixed specimens.

Introduction

Fluorescent indicators are essential tools for studying the structure and function of the nervous system (Feng et al. 2000; Gan et al. 2000; Giepmans et al. 2006). For example, carbocyanine dyes, due to their high extinction coefficients and lipophilic properties, have been very useful for labeling neurons in their entirety and for tracing long-range neuronal connections. These dyes are maintained within the cell membrane and can be imaged for extended periods of time without obvious cytotoxicity (Honig and Hume 1986; Liu and Westerfield 1990). In addition, fluorescent calcium indicators have been critical for monitoring calcium dynamics in neuronal processes and studying the role of calcium signaling in neuronal plasticity (Giepmans et al. 2006; Garaschuk et al. 2007).

Loading fluorescent indicators into individual cells is often accomplished with sharp microelectrodes or patch-pipettes (Neher and Almers 1986; Liu and Westerfield 1990; Gan and Macagno 1995a; Gan and Macagno 1995b; Gan et al. 1999). Such dye-loading approaches are technically demanding and time consuming, and only a small number of cells can be labeled at any one time. Here we describe a detailed protocol for particle-mediated ballistic delivery of fluorescent dyes into individual cells. This technique allows rapid labeling of entire neurons or glia in a Golgi-like manner after membranes of individual cells are contacted by particles coated with lipophilic dyes (Gan et al. 2000). Many cells can be labeled by dyes of different colors at controlled densities to facilitate the study of structural interactions between cells (Gan et al. 2000; Rockhill et al. 2002; Strettoi et al. 2002; Sun et al. 2002; Kong et al. 2005; Coombs et al. 2007; Li et al. 2009; Yu et al. 2009).

Furthermore, in conjunction with other histochemical labeling methods, the technique can be used to study neuronal/glial structures associated with pathologic processes in animal models or postmortem human brain (Alifragis et al. 2002; Grutzendler et al. 2003; Moolman et al. 2004; Oberheim et al. 2008; Neely et al. 2009). In addition to lipophilic dyes, water-soluble molecules such as calcium indicators can also be delivered efficiently with this technique (Kettunen et al. 2002; Lohmann et al. 2002; Lohmann and Bonhoeffer 2008). The ballistic delivery of indicators into individual cells thus provides a useful approach to study the structure and function of the nervous system.

Imaging Setup

A confocal microscope is recommended for imaging fluorescently-labeled cells. We frequently use an oil immersion objective 60×1.4 NA for high-resolution imaging of neuronal structures such as dendritic spines.

Materials

Reagents

Calcium indicators (e.g., Calcium Green-1 dextran, Molecular Probes)

Lipophilic dyes: DiI, DiO or DiD (Molecular Probes, Cat# D-282, D-275, D-307).

Methylene chloride (Sigma).

Polyvinyl-pyrrolidone (PVP, 10mg/ml in distilled water, from Sigma or Bio-Rad)

Equipment

Biolistic device: Helios Gene Gun System (Bio-Rad) or other custom made ballistic devices (see <http://thalamus.wustl.edu/nonetlab/ResourcesF/genegun/Genegun.htm>).

Glass slides

Isopore polycarbonate membrane filter with $3 \mu\text{m}$ pore size and 8.0×10^5 pores/cm² density (Cat #. TSTP04700, Millipore).

Plastic Petri dish

Sonicator

Tefzel tubing (Bio-Rad; Cat# 165-2411)

Tungsten particles (1.0 - $1.7 \mu\text{m}$ diameter, Bio-Rad)

Tubing prep station (Bio-Rad; Cat #165-2418)

Experimental Method

Ballistic Delivery of Lipophilic Dyes for Labeling Cells

Preparation of Stock Solutions/Lipophilic Dyes—1. For single color labeling, dissolve 3 mg of lipophilic dye such as DiI, DiO or DiD in 100 μl of methylene chloride. For multicolor labeling, dissolve 7 mg of DiI, DiO and DiD in 70 μl of methylene chloride in three separate microfuge tubes. Mix various proportions of the different dyes to obtain, for example, 7 different dye combinations in 7 tubes as follows: (a) 30 μl DiI; (b) 30 μl DiO; (c) 30 μl DiD; (d) 15 μl DiI: 15 μl DiO; (e) 15 μl DiI: 15 μl DiD; (f) 15 μl DiO: 15 μl DiD; (g)

10 μ l DiI: 10 μ l DiO: 10 μ l DiD. Last, add 70 μ l of methylene chloride to each tube. The final concentration for each dye combination is 3 mg in 100 μ l of methylene chloride.

Coating Tungsten Beads—2. Spread tungsten particles (50 to 100 mg) evenly on a glass slide by mixing with a few drops of methylene chloride. Then add the dye solution (100 μ l) to the particles on the glass slides. As methylene chloride evaporates quickly (within a minute), the lipophilic dye precipitates onto the tungsten particles. Scrape the dye-coated particles off the glass slide using a razor blade and collected in a 10 ml test tube. For multicolor labeling, coat particles separately with different combinations of dyes and collect them together in a 10 ml test tube. Resuspend the particles in 3-10ml of distilled water and sonicated for 5-10 minutes to prevent formation of large clusters.

Bullet Preparation—3. Inject sonicated solutions of particles of single color or mixtures of colors into a 30 inch long tefzel tube. To improve attachment of the beads to the tube walls, introduce a solution of polyvinyl-pyrrolidone (PVP, 10mg/ml in distilled water) and rapidly drain to pre-coat the tube prior to injecting the dye solutions. Then insert the tube into a “tubing prep station” (Bio-Rad) and allow the dye-coated particles to precipitate and settle onto the tube wall for ~3 minutes (depending on the desired labeling density) before slowly withdrawing the remaining liquid. To spread the beads evenly, rotate the tube on the “prep station” for ~5 minutes and dry with a constant air flow. Cut the air-dried tube into 13 mm pieces and store at room temperature, protected from light, for future use.

Particle Delivery—4. Deliver dye-coated particles to the preparation using a commercially available biolistic device (“gene gun”, BioRad). Such a particle delivery device can also be custom-made at much less expense (see <http://thalamus.wustl.edu/nonetlab/ResourcesF/genegun/Genegun.htm>). To prevent large clusters of particles from landing on the tissue, causing non-uniform labeling and interfering with single-cell resolution, it is useful to insert an Isopore polycarbonate membrane filter between the gene gun and the preparation (see below). A filter holder can be made by cutting a central hole (~20 mm diameter) on the bottom of a plastic Petri dish and covering it with a metal mesh (~1mm pore) glued to it. A membrane filter is then placed on top of the metal mesh and held immobile by using a Petri dish cover also with a central opening of ~20mm diameter (Fig. 1). The filter holder is then placed on top of the target tissue and aligned with the gene-gun barrel prior to shooting (Fig. 1).

Labeling Density and Depth—5. The labeling efficiency is dependent on multiple factors such as bullet particle density, filter pore density, gun pressure and distance from the tissue. To reduce the labeling density, a porous polycarbonate membrane is usually inserted between the “gun” and the target tissue. This prevents large clusters of dye-coated particles from landing on the preparation and causing diffuse dye labeling (Fig. 1C). The use of a membrane filter also protects the tissue from the air shock wave generated by the gun. Labeling efficiency can be increased either by producing bullets with more dye per particle or by shooting the preparation multiple times.

Tissue Fixation Conditions—6. The fixation condition is a critical variable in obtaining good quality labeling of neuronal structures. For mouse brain slices, perfuse the animal with 10 ml of isosmotic PBS followed by rapid perfusion with 40 ml of 4% paraformaldehyde in PBS (20 ml/min). Remove the brain quickly from the skull and post-fixed in 4% paraformaldehyde for 10 min. Label vibratome-cut brain slices (200-300 μ m thickness) with the ballistic method, and post-fix for 2-12 hours at room temperature to further preserve structures and allow dye diffusion.

Dye Diffusion and Tissue Mounting—7. After delivery of particles, keep tissues such as brain slices in solution (e.g., 0.2M PBS) for several hours to allow dye diffusion along neuronal processes. In fixed tissue labeling takes longer than in living slices presumably because of slower dye diffusion along the fixed plasma membrane. Following dye diffusion, mount tissues on glass slides using 80% glycerol in 0.2M PBS or 100% glycerol.

Labeling with water-soluble dyes

The following modifications to the protocol are required for the delivery of water-soluble dyes: *a.* Dissolve 1~2 mg of a dye, such as Calcium Green-1 dextran in 12-25 μ l of distilled water. *b.* Spread 25~50 mg of tungsten particles (1.3 or 1.7 μ m diameter) evenly onto a clean microscope glass slide. Use a razor blade to dissociate large clusters of particles until a fine powder results. *c.* Pipette the dissolved dye solution uniformly onto the tungsten particles and air-dry. *d.* Scrape the dye-coated particles off onto a piece of weighing paper with the razor blade. *e.* Pour the powder of dye-coated particles (in a dry form unlike in the case of lipophilic dye loading) into a 15 cm long tefzel tubing, pre-coated with polyvinylpyrrolidone (10 mg/ml in distilled water). Gently rotate and shake the tube until the particles spontaneously adhere onto the inner wall. Cut the particle-coated tube into 13 mm pieces and store in a dark, dry container at 4°C until use. *f.* For particle delivery into the tissues, we use virtually the same protocol as the one described for lipophilic dyes.

When using water-soluble dyes, labeling density needs to be approximately 5-fold higher than with lipophilic dyes to increase the probability of successful labeling because dyes need to enter the cell soma and diffuse into the cytoplasm rather than diffuse along the cell membrane as with lipophilic dyes. Because of this, labeling with water-soluble dyes works best in preparations containing high density of cell somata such as the ganglion cell layer of the retina in which 15% of cells can be routinely labeled.

Troubleshooting

Problem: No fluorescently labeled cells were found.

Steps 2 and 4

Solution: First, check if dye-coated tungsten particles were delivered to the preparation. If not, re-shoot to deliver enough particles to the preparation. If there were many tungsten particles but no fluorescently-labeled cells on the surface of the preparation, check if tungsten particles were coated with enough fluorescent indicators. If not, prepare new bullets to make sure that tungsten beads are coated with enough dyes.

It is important to point out that density of labeling can be controlled by using various gas pressures (60-200 psi Helium gas), by changing the distance between the gun and the preparation (10-25 mm) or by shooting the tissue several times. Lower gas pressures and larger distance between the gun and the tissue lead to lower labeling densities. Higher gas pressures may cause membrane filters to break apart, and as a result, tissue injury and non-uniform labeling may occur.

Problem: Neuronal processes are not clearly labeled.

Step 6.

Solution: Check the fixation conditions. The duration of fixation prior to ballistic particle delivery is important for obtaining good quality labeling. Over-fixation prior to shooting (>1h) often causes dye to spread between cells or nearby processes, and thus reduces the number of cells that can be individually resolved. However, after ballistic labeling, the

duration of post-fixation does not appear to cause dye spread between nearby cells, suggesting that dye spread and decreased labeling is probably due to failure of initial dye transfer from the particle to surrounding individual cells. The problem of dye spread between cells due to over-fixation is less severe in young animals, presumably because of greater extracellular space.

Fixation conditions are also critical when labeling post-mortem human brain. It appears that labeling is best when small pieces of brain are fixed with 4% paraformaldehyde within 24 h post-mortem. Because lipophilic dye labeling requires the presence of an intact and continuous cell membrane, it is important to avoid using fixatives with substances that can dissolve membrane lipids such as methanol contained in standard formalin. In addition, frozen specimens cannot be labeled by the lipophilic dyes because of the membrane disruption that occurs during the freezing and thawing process.

Discussion

The ballistic technique has been used to label neurons in both live and fixed tissues, ranging from isolated neurons in culture to whole brain and brain slices (Fig. 2). When labeling at low densities, we typically observed only one dye-coated particle contacting either the soma, or a branch of the dendritic or axonal arbor of a labeled cell, indicating that a single particle contains enough dye to label one cell through retrograde and anterograde diffusion (Fig. 2a-b). Fig. 2c shows multiple pyramidal neurons labeled in a fixed mouse cortical brain slice by delivering a mixture of particles coated with different combinations of dyes. Many neurons and their processes, including dendritic spines, appeared completely labeled and can be distinguished from each other due to the multicolor labeling. Labeled dendritic arbors and axons can often be followed for hundreds of micrometers. In living tissues, dendritic processes appeared labeled almost immediately (<5 minutes) after particles contacted the tissue (Gan et al. 2000; Kettunen et al. 2002; Grutzendler et al. 2003). The technique is especially useful for morphological analysis of neurons in retinal explants (Gan et al. 2000; Rockhill et al. 2002; Strettoi et al. 2002; Sun et al. 2002; Kong et al. 2005; Coombs et al. 2007) and has also been used for analysis of dendritic spines in different regions of the brain (Grutzendler et al. 2003; Tsai et al. 2004; Wu et al. 2004a; Wu et al. 2004b; O'Brien and Unwin 2006). Furthermore, it has also been successfully used for morphological analysis of astrocytic domains in both mouse and post mortem human brain (Oberheim et al. 2008; Oberheim et al. 2009).

In addition to lipophilic dye labeling, the ballistic technique can be used to label cells with water-soluble compounds such as calcium indicators (Kettunen et al. 2002; Lohmann et al. 2002; Lohmann and Bonhoeffer 2008). Fig. 2d shows a cortical pyramidal cell from an acute brain slice labeled successfully upon particle delivery of calcium indicators such as Calcium Green-1. The time required for complete filling of dendritic arbors occurred within a minute. The intensity of labeling varied from cell to cell, most likely because particles carried differing amounts of indicator. In addition, these labeled cells demonstrated a rise in intracellular calcium levels upon stimulation with KCl, indicating that they remained viable after labeling (Kettunen et al. 2002; Lohmann et al. 2002).

The ballistic method allows rapid delivery of fluorescent indicators into multiple cells types in both fixed and living preparations, ranging from embryonic tissue to adult postmortem human brain. This labeling technique provides several advantages over existing labeling methods:

a. Efficient labeling of multiple cells

In comparison to intracellular injection of indicators using micropipettes, this approach allows labeling of many neurons within minutes rather than targeting cells individually. Dendrites in many cells are often labeled from a single indicator-coated particle and the labeling process appears not to cause a significant perturbation of cellular structure or function (Gan et al. 2000; Kettunen et al. 2002; Lohmann et al. 2002). In addition, ballistic delivery can be very useful in immature cells in which dye injection through micropipettes is extremely difficult because of cell fragility and susceptibility to damage. Finally, using various combinations of carbocyanine dyes, adjacent neurons or and glia can be labeled in different colors (Gan et al. 2000; Oberheim et al. 2008). Combined with high density labeling, this multicolor feature is potentially useful for studying complicated neuronal networks.

b. Rapid labeling

The ballistic technique permits immediate imaging of neuronal tissues as soon as the preparation is made, unlike GFP transfection techniques that require many hours for gene expression (Lo et al. 1994). This is particularly important for brain slice experiments where the health of the preparation can only be maintained for a limited period of time. In addition, the technique allows extensive dendritic arbors to be labeled rapidly without dialyzing cellular contents as seen in patch pipette loading (Majewska et al. 2000).

c. Labeling a variety of cell types in live and fixed tissues

The labeling technique involves passive dye transfer and diffusion that is independent of gene transcription and protein synthesis. Thus, any type of cells in fixed or living tissues can be labeled non-selectively. Furthermore, more complete labeling of fine neuronal structures such as dendritic spines is achieved with lipophilic dyes compared to immunolabeling.

d. Labeling with various indicators and molecules

In addition to delivering lipophilic dyes and calcium-indicators as described here, the ballistic technique is potentially useful for delivering other indicators and molecules, such as voltage-sensitive dyes, dextran conjugated pH indicators, ion indicators and pharmacological reagents. This potential was exploited in recent studies using a rhodamine analogue to investigate cell migration in embryonic brains (Alifragis et al. 2002). It is also possible to simultaneously deliver more than one indicator by co-coating the particles with multiple substances (O'Brien and Lummis 2004).

e. Combining the ballistic labeling technique with other labeling approaches

The ballistic labeling technique in combination with other fluorescent dyes such as Thioflavin-S has been used to study dendritic abnormalities near amyloid plaques in a mouse model of Alzheimer's disease and in human brain (Grutzendler et al. 2003). In addition, if immunostaining does not require extensive tissue permeabilization and lipid extraction, antibody labeling can also be used in conjunction with lipophilic dye labeling (Alifragis et al. 2002; Neely et al. 2009).

Despite many advantages, ballistic delivery of indicators also has some drawbacks: First, this approach is highly variable because dye crystal size, density and penetration are difficult to control. Using the commercially available Bio-Rad "gene gun", the depth of penetration of dye-coated particles is generally ~20-30 μm and up to 60 μm . Therefore, most of the labeled cells are usually located near the surface. However, because of retrograde transfer of dyes, cells located deeper can also be labeled, particularly with lipophilic dyes. A potentially useful modification to the gene gun that can increase the depth of penetration substantially

was reported (O'Brien et al. 2001). Such a modification may allow penetration of particles up to ~300 μm in brain slices. Second, because water-soluble dye-coated particles have to be in or near the cell somata for labeling to occur, it is difficult to get high density labeling without showering the tissue with many particles and potentially leading to some tissue damage. Third, whereas labeling of dendritic structures is very good, especially with lipophilic dyes, labeling of axonal arbors over extensive distances has not been successful. This is likely because each particle carries a limited amount of dye on its surface. In the future, it may be possible to improve labeling by designing special beads that can carry a larger amount of dye. Another limitation of the technique is the difficulty in targeting a particular cell or region. However, using a mask to cover parts of the preparation during shooting one can prevent labeling of undesired areas and target a smaller region of interest.

In summary, we describe here in detail a ballistic technique that delivers fluorescent dyes and indicators into multiple cell types, providing rapid labeling of neurons, glia and their processes both in vivo and in vitro. This approach should be useful for studying neuronal connectivity, function and pathology in the nervous system of experimental animals as well as in post-mortem human tissue specimens.

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Fig. 1. Membrane filter holder. a). From left to right: metal mesh glued to a Petri dish with 20 mm diameter opening, membrane filter, a Petri dish cover with ~20 mm diameter opening. b). membrane filter is placed on top of metal mesh and held in place by the Petri dish cover. c). Membrane filter holder is placed on top of tissue and aligned with the gene gun barrel.

Fig. 2.

Ballistic labeling of cells in fixed and living tissue. An individual cell in a P10 mouse brain slice is labeled with lipophilic dye with a single dye-coated particle (arrow) landed on either the cell soma (a) or a dendritic arbor (b). c). Labeling of many pyramidal neurons from a fixed brain slice from a P20 mouse that was shot with a combination of seven different lipophilic dyes. The image represents a collapsed view of 50 confocal planes covering approximately 25 microns of depth. Many of the cells had processes that extended beyond the upper or lower surface of the slice and hence have dendritic or axonal branches that end abruptly. d). Labeling of a pyramidal neuron and spines (inset) with Calcium Green-1 dextran from an acute slice preparation of a P12 rat. Scale Bar, 10µm for a-d.